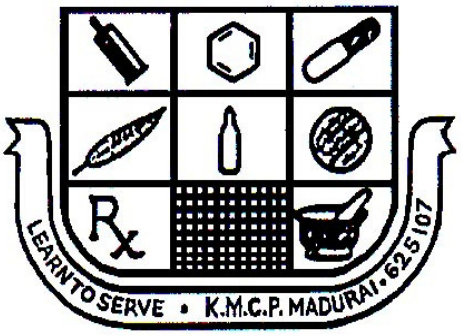
METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR BY REVERSE PHASE LIQUID CHROMATOGRAPHY

Dissertation submitted in partial fulfillment of the requirement for the award of the degree of

MASTER OF PHARMACY

Of

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY, CHENNAI.



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

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Completing a task is never a one man effort. It is often a result of invaluable contributions of a number of individuals in a direct or indirect manner. This suitably applies to my dissertation work.

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With This I Remain,

Naresh Rayapati

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CERTIFICATE

This is to certify that the project entitled **"METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF TENOFOVIR BY REVERSE PHASE LIQUID CHROMATOGRAPHY"** by ***NARESH RAYAPATI (Reg. No. 26093126)*** in partial fulfillment of the degree of Master of Pharmacy in Pharmaceutical Analysis under Tamil Nadu Dr. M. G. R Medical University, Chennai, done at **K. M. COLLEGE OF PHARMACY,** MADURAI-625107, is a bonafide work carried out by him under my guidance and supervision during the academic year SEPTEMBER-2010-2011. The dissertation partially or fully has not been submitted for any other degree or diploma of this university or other universities.

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LIST OF ABBREVIATIONS USED

ACN	-	Acetonitrile
BP	-	Boiling point
LOD	-	Limit of detection
LOQ	-	Limit of quantification
cm	-	Centimeter
Conc	-	Concentration
GC-MS	-	Gas chromatography- mass spectroscopy
gm	-	Grams
HPLC	-	High performance liquid chromatography
HPTLC	-	High performance thin layer chromatography
ICP-MS	-	Inductive coupled plasma- mass spectroscopy
IS	-	Internal standard
LC-MS	-	Liquid chromatography- mass spectroscopy
LOD	-	Limit of detection
LDL	-	Low-density lipoprotein
LOQ	-	Limit of quantitation
mg	-	Milligrams
min	-	Minutes
ml	-	Millilitre
mM	-	Millimolar
mm	-	Millimeter
MW	-	Molecular weight
µg	-	microgram
µl	-	microlitre
µm	-	micrometer
ng	-	Nanogram
NH_2	-	Amino

nm	-	Nanometer
NLT	-	Not less than
NMT	-	Not more than
pH	-	Negative logarithm of hydrogen ion
pKa	-	Dissociation constant
psi	-	per square inch
RI	-	Refractive index
RP-HPLC	-	Reverse phase high performance liquid chromatography
RSD	-	Relative standard deviation
SD	-	Standard deviation
Sl. No	-	Serial number
Std	-	Standard
THF	-	Tetrahydrofuran
TLC	-	Thin layer chromatography
UV	-	Ultra-violet
USP	-	United states pharmacopoeia
Vs	-	Versus
v/v	-	volume/volume
λ	-	Lambda
ºC	-	degree centigrade
%	-	Percentage

CONTENTS

1. INTRODUCTION

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

INTRODUCTION [1]

HPLC was introduced commercially in 1969 and since then it has undergone extensive modifications and innovation, which lead to its emergence as the foremost analytical tool for quantitative analysis. HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain a satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

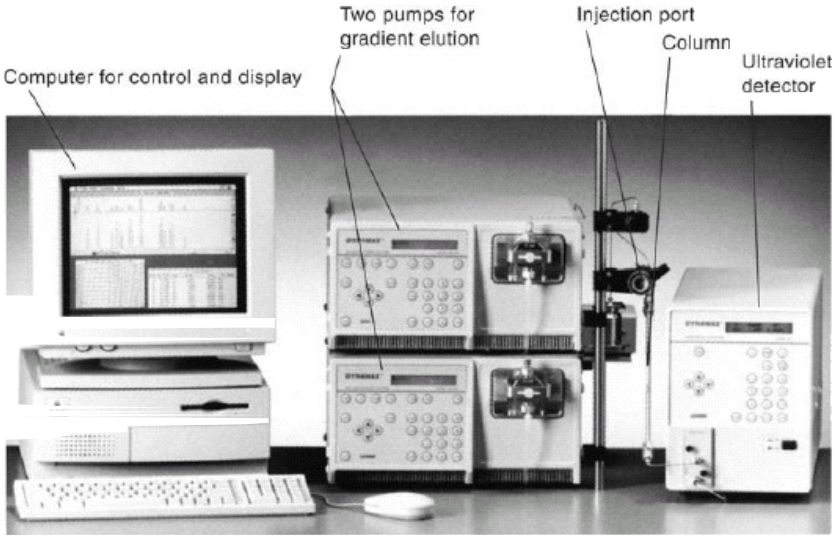


Fig.1 HPLC System.

The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process. If diffusion is minimized, a faster and effective separation can be achieved. The technique of HPLC is so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages,

- Speed (many analysis can be accomplished in 20 min or less)
- Greater sensitivity (various detectors can be employed)
- Improved Resolution (wide variety of stationary phases)
- Reusable columns
- Ideal for the substances of low volatility
- Easy sample recovery, handling and maintenance
- Instrumentation leads itself to automation and quantization
- Precise and Reproducible
- Calculations are done by integrator itself

TYPES OF HPLC TECHNIQUES:

Based on modes of chromatography:

- Reverse phase chromatography
- Normal phase chromatography

Based on principle of separation:

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elution technique:

- Isocratic separation
- Gradient separation

Based on the scale of operation:

- Analytical HPLC
- Preparative HPLC

Reversed phase chromatography

In 1960s, chromatographers started modifying the polar nature of the silanol group by chemically reacting silicon with organic silanes.

The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica in now reversed i.e., it is non-polar or the nature of the phase is reversed, the chromatographic separation carried out with such silica is referred to as reversed-phase chromatography.

A large number of chemically bonded silica based stationary phases are available commercially. Silica based stationary phases are still more popular in reversed phase chromatography; however other adsorbents based on polymer (styrene divinyl benzene copolymer) are slowly gaining ground.

The less water-soluble compounds are better retained by the reversed phase surface. The retention time decreases in the following order: Aliphatic > induced dipoles (E.g. CCl_4) > permanent dipoles (E.g. $CHCl_3$) > weak Lewis bases (Ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases.

As a general rule, the retention increases with an increase in the contact area between sample molecule and stationary phase i.e., with an increase in the number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase system the strong attractive forces between water molecules arising from the 3-dimensional intermolecular hydrogen bonded network present in the structure of water must be distorted or disrupted when a solute is dissolved.

Only higher polar or ionic solutes can interact with the water structure. Now polar solutes are squeezed out of the mobile phase and are relatively insoluble in it, but with the hydrogen carbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) and alkane with 18 carbon atoms is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reverse phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly and highly with silanol groups, so that adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl group such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

Adsorption chromatography / normal phase chromatography

In normal phase chromatography, the stationary phase is polar adsorbent. The mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol group at the end in normal phase separations. These OH groups are statistically distributed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase.

This forms a weak bond with many molecules in the vicinity when any of the following interactions are present. Dipole-induced dipole, dipole-dipole, hydrogen bonding, -complex bonding. These situations arise when the molecule has one or several atoms with lone pair electrons or a double bond. The adsorption strengths and hence ‘K’ value (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatic < organic < halogen compounds < sulphides < ethers < esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional

groups in the sample molecule but also on stearic factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as aminopropyl, cyanopropyl and diol phases are the stationary phases alternative to silica gel in normal phase chromatography.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phase and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in the increased homogeneity of the stationary phase surface.

Polar modifiers such as acetic acid or triethylamine (TEA) are added to the mobile phase, to deactivate the more polar adsorption sites on the surface of stationary phase, which in turn will improve peak shape as well as the reproducibility of the retention times.

Ion-exchange Chromatography

Separation is based on the charge-bearing functional groups, anion exchange for sample negative ion, or cation exchange - for sample positive ion. Gradient elution by pH is common.

Size Exclusion Chromatography

Also known as gel permeation or filtration, separation is based on the molecular size or hydrodynamic volume of the components. Molecules that are too large for the pores of the porous packing material on the column elute first, small molecules that enter the pores elute last and the elution rates of the rest depend on their relative sizes.

Affinity/ Ion-pair Chromatography

Separation is based on a chemical interaction specific to the target species. The more popular reversed phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvent(s). Affinity chromatography, common for macromolecules, employs a ligand (biologically active molecule bonded covalently to the solid matrix) which interacts with

its homologous antigen (analyte) as a reversible complex that can be eluted by changing buffer conditions.

Chiral Chromatography:

Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on chiral stationary phases. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug.

Isocratic Separation:

In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained the process.

Gradient Separation:

In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

Analytical HPLC:

In this only analysis of the samples are done. Recovery of the samples for reusing is normally not done, since the samples used are very low.

Preparative HPLC:

Where analysis of the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused.

INSTRUMENTATION

PUMPS [2]

The HPLC pump is considered to be one of the most important components in a liquid chromatography system which has to provide a continuous constant flow of the eluent through

the HPLC injector, column and detector. Pumps are constructed with materials such as stainless steel, Teflon, Glass.

Standard HPLC Pump requirements are

Flow Rate Range : 0.01 – 10 ml/min

Pressure Range : 1 – 5,000 psi

Pressure Pulsations : Less than 1% for Normal and Reverse phase HPLC

Less than 0.2%. for Size Exclusion Mode.

The two basic classifications are Constant Pressure Pump and Constant Flow Pump. The first is used for column packing and the second type is most widely used in all common HPLC applications.

Constant Pressure pump can deliver a constant flow rate if the pump operates against a constant column backpressure and if the viscosity of the mobile phase remaining constant. Constant pressure pump may be simple gas displacement pump or pneumatic amplifier pump. Simple gas displacement pump is a reservoir such as coil of tubing to which pressure is applied from a gas cylinder. The disadvantage of this pump is limited solvent capacity. Pneumatic amplifier pump is a modification of simple gas displacement pump. The gas pressure is applied to a large piston, which is connected to a small diameter piston, which is in contact with mobile phase when all the mobile phase is used up, the piston returns quickly by pneumatic means, thus refilling the chamber. The advantage of this is pulse less flow and unlimited solvent capacity.

POSITIVE DISPLACEMENT (SYRINGE) PUMP

Syringe pump is a single stroke displacement pump which has to be refilled after it displaces the whole syringe volume. On the other hand, a syringe pump does not have any flow and pressure pulsations compared to the reciprocating pump. A screw fed drive connected to a stepping motor actuates the piston inside the chamber. For the micro-HPLC applications a syringe pump allows for the maintaining of a constant flow at the micro liter per minute flow rate range and requires no check valves.

Mixing unit is used to mix solvents in different proportions and pass through the column. The two primary methods of blending the mobile phase components are Low Pressure Mixing and High Pressure Mixing.

In an isocratic separation, mobile phase is prepared by using pure solvent or mixture of solvents, i.e., solvent of same eluting power or polarity is used. But in gradient elution technique, the polarity of the solvent is gradually increased and hence the solvent composition has to be changed. Hence a gradient controller is used when two or more solvent pumps are used.

INJECTORS

Injectors for liquid chromatographic systems should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi). They should also produce minimum band broadening and minimize possible flow disturbances. Generally, the most useful and widely used sampling device for modern LC is the micro sampling injector valve.

RHEODYNE INJECTOR

Because of their superior characteristics, valves are now used almost to the exclusion of syringe injection. With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow, even at elevated temperature.

AUTOMATIC INJECTORS

With commercially available automatic sampling devices, large number of samples can be routinely analyzed by LC without operator intervention. Such equipment is popular for the analysis of routine samples (e.g., quality control of drugs), particularly when coupled with automatic data-handling systems. Automatic injectors are indispensable in unattended searching (e.g., overnight) for chromatographic parameters such as solvent selectivity, flow rate, and temperature optimization. Most of the auto samplers have a piston metering syringe type pump to suck the reestablished sample volume into a line and then transfer it to the relatively large loop (~100 ml) in a standard six-port valve. The simplest auto samplers utilize the special vials with

pressurization caps. A special plunger with a needle, push the cap down to the vial and displace the sample through the needle into the valve loop. Most of the auto samplers are microprocessor controlled and can serve as a master controller for the whole instrument.

COLUMNS

HPLC column are made up of stainless steel or glass, which differs in length and inside diameter depending on the application. The two types of columns are Analytical column and Preparative column. Standard analytical columns are 4-5 mm in internal diameter and 10-30 cm in length. The particle size used ranges from 5-10 pm. Preparative columns are 20-50 mm in internal diameter and 20-100 cm in length. The particle size used ranges from 37-50 ·m.

PRE COLUMN FILTERS: (Guard Column)

Prevents the contamination of the expensive analytical columns with fine particles that can eventually clog the mobile phase flow. Made up of Porous stainless Frit (0.5 – 2 ·m) or a little piece of sacrificial column.

Functional group present in stationary phase depends on the type of chromatographic separation. In normal phase mode it contains the silanol groups (OH group), in reverse phase mode it contains Octyl Decyl Silane (ODS) column, Octyl column, Butyl column, Nitrile column, Amino column, Base deactivated silane (BDS) column etc., for other modes of chromatography, ion exchange, gel columns, and chiral columns are available.

DETECTORS

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. Generally, there are two types of HPLC detectors, bulk property detectors and solute property detectors.

a) Bulk property detectors:

These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity and dielectric constant detectors.

b) Solute property detectors:

Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase. These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase before the detection. Solute property detectors which do not require the removal of the mobile phase before detection include spectrophotometric (UV or UV-Visible) detector, fluorescence detectors, polarographic, electro-chemical and radio activity detectors, where flame ionization detector and electron capture detector both require removal of the mobile phase before detection.

RECORDERS AND INTEGRATORS

Recorders are used to record the responses obtained from detectors after amplification, if necessary. They record the baseline and all the peaks obtained, with respect to time. Retention time for all the peaks obtained, with respect to time. Retention time for all the peaks can be found out from such recordings, but the area of individual peaks cannot be known.

Integrators are improved version of recorders with some data processing capabilities. They can record the individual peaks with retention time, height and width of peaks, peak area, percentage of area, etc. integrators provide more information on peaks than recorders. Nowadays computers and printers are used for recording and processing the obtained data and for controlling several operations.

APPLICATIONS IN PHARMACEUTICAL ANALYSIS

The sample or solute is analyzed quantitatively in HPLC by either peak height or Peak area measurements. Peak areas are proportional to the amount of constant rate. Peak heights are proportional to the amount of material only when peak width is constant and are strongly

affected by the sample injection techniques. Once the peak height or the peak areas are measured, there are five principle evaluation methods for quantifying the solute.

Calibration by Standards

Calibration curves for each component are prepared from pure standards, using identical injection volumes and operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear.

Concentration of solute = Proportionality Constant (k) x Area

In this evaluation method only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of a given detector differs for each molecule type of compounds.

Internal Standard Method

In this technique a known quantity of the internal standard is chromatographed and area Vs concentration is ascertained. Then a quantity of the internal standard is added to the raw sample prior to any sample pretreatment or separation operations.

The peak area of the standard in the sample run is compared with the peak area of the standard run separately. This ratio serves as a correction factor for variation in sample size, for losses in any preliminary pretreatment operations or for incomplete elution of the sample. The material selected for the internal standard must be completely resolved from adjacent sample components must not interfere with the sample components and must never be present in samples.

Standard Addition Method

If only few samples are to be chromatographed, it is possible to employ the method of standard addition(s). The chromatogram of the unknown is recorded, then a known amount of analyte(s) is added and the chromatogram is recorded using same reagents, instruments and other conditions. From the increase in the peak area (or peak height), the original concentration can be computed by interpolation.

The detector response must be a linear function of analyte concentration and yield no signal at zero concentration of the analyte. Sufficient time must elapse between addition of the standard and actual analysis to allow equilibrium of added standard with any matrix interfering.

A correction for dilution must be made if the amount of standard added changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.

External Standard Method

It employs a separate injection of a fixed volume of sample and standard solution. The peaks are integrated and concentration is calculated.

The selection of suitable chromatographic (HPLC) system for a given mixtures of solutes cannot be made with certainty and must be confirmed by experiment. If the chemical nature of the sample components is known, then the phase system can be selected from the literature references. If nothing is known about the chemical nature of sample, then the sample solubility will give some indication as to which chromatographic method to employ.

Best column, best mobile phase, best detection wavelength, efforts in their selection can make a world of difference while developing HPLC method for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results and validated method for separation.

INTRODUCTION TO METHOD DEVELOPMENT [(3)]

HPLC method development for the analysis of mixtures of substances is a task that usually requires much expertise. It is also extremely time-consuming. In spite of advances in chromatographic theory, HPLC method development is still based mainly on “trial and error”.

The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method. Method development in Liquid chromatography usually requires selecting the method requirements and deciding what type of instrument to utilize and why. In the development stage,

decisions regarding choice of column, mobile phase, detectors, and method of quantitation must be addressed.

The following are the reasons for developing new methods of analysis:

- The method may not be suitable for particular analyte.
- Existing method may be too error or contamination prone or they may be unreliable.
- Existing method may be too expensive, time consuming or energy intensive, or they may not be easily automated.
- Existing method may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including improved analyte identification or detection limits, greater accuracy or precision or better return on investment.
- There may be a need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

The goals of the method are necessary to translate into a method development design. Goals for new or improved analytical methods might include the following.

- Qualitative identification of the specific analyte(s) of interest providing some structural information to confirm "general behavior"(e.g. retention time, colour change, pH etc.,
- Quantitative determination, at trace levels when necessary that is accurate, precise, and reproducible in any laboratory setting when performed according to established procedures.
- Ease of use, ability to be automated, high sample throughput, and rapid sample turn around time.
- Decreased cost per analysis from using simple quality assurance and quality control procedures.
- Preparation of samples that minimize time, effort, materials, and volume in of sample consumed.

The first step in the method development process is to decide which chromatographic method will be used. Choices may be as diverse as GC, LC or superficial-fluid chromatography (SFC), Normal – phase (NPLC) or reverse phase chromatography (RPLC), and ion-exchange

(IEC) or ion-pairing chromatography (IPC). Within RPLC, silica modified with octadecyl, Octyl or short alkyl chains or with phenyl, amino or Cyano groups may form the stationary phase. This shows that there is a 'selection tree' for chromatographic systems. For each stationary phase there may still be a number of different mobile phases from which to select the most promising starting conditions (or the best 'first guess').

Steps involved in HPLC Method Development:

Step-1:

Method Selection: The first step in developing an HPLC method is to always consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions-this will save time, doing unnecessary experimental work. The information from literature should contain.

- Solubility profile – which provides the information about the solubility of drug substances in different solvents and at different pH conditions.
- Analytical profile – which provides the information about the analytical profile of the drug substance, impurities and degradation products.
- Stability profile – which provides the information about the stability profile of the drug substance with respect to storage conditions.

Step-2

Retention Optimization: This step determines the optimum conditions to adequately retain all analytes that ensures no analyte has a capacity factor limits not less than 0.5 and not greater than 10-15.

- Poor retention could result in peak overlapping.
- Excessive retention leads to long analysis time and broad peaks with poor detection.

Step-3

Selectivity Optimization: The mobile phase and stationary phase compositions need to be taken into account to achieve adequate selectivity (peak spacing). To minimize the number of trail chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. To select these, the nature of the analytes must

be considered. It is much easier to optimize mobile phase parameters than to optimize stationary phase.

Step-4

System Optimization: This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column packing, particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step-5

Method Validation: Proper validation of analytical methods is important for pharmaceutical analysis when ensuring of the continuing efficacy and safety of each batch manufactured relies solely in the determination of quality. Method Validation is generally a one-time process performed after the method has been developed to demonstrate that the method is scientifically sound and that serves the intended analytical purpose.

METHOD VALIDATION PARAMETERS [4]

ICH for registration of pharmaceuticals has developed consensus text on validation of analytical procedures which include,

Specificity

It is the ability to asses' unequivocally the analyte in the presence of components which may be expected to present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

Procedures used to demonstrate specificity will depend on the intended objective of the analytical procedures.

It is not always possible to demonstrate that analytical procedures is specific for a particular analyte (complete discrimination).In this case a continuation of two or more analytical procedure is recommended to achieve necessary level of discrimination.

Accuracy

Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as conventional true value or on an accepted reference value and the value found.

Assay

Active substance

Several methods of determining accuracy are available

a. Application of analytical procedures to an analyte of known purity (Eg.Reference material).
b. Comparison of the results of the proposed analytical procedures with those of a second well-characterized procedure, the accuracy of which is stated and /or defined.
c. Accuracy may be inferred once precision linearity and specificity has been established.

Medicinal products - methods include

a. Application of analytical procedure to synthetic mixtures of product components to which known quantities of substance to be analyzed have been added.
b. In case where it is impossible to obtain samples of all product components, it may be acceptable either to add known quantities of the analyte to the product or to compare the result obtained from a second well characterized procedure, the accuracy of which is stated and defined.

Impurity (Quantification) [5]

Accuracy should be assessed on sample (substance/products) spiked with known amount of impurities

Precision

The precision of analytical procedure express the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Precision of an analytical procedure is usually expressed at the variance, standard deviation or coefficient of variation of series of measurements.

Repeatability

Express the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra assay precision.

It should be assessed using a minimum of nine determinations covering the specified range for the procedure (Eg. three concentration /three replicates each) or a minimum of determination at 100% of the test concentration.

Intermediate precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. Typical variations to be studied include days, analysts, equipments etc.

It is not necessary to study these effects individually. The use of experimental design (matrix) is encouraged.

Reproducibility

Reproducibility is assessed by means of an interlaboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance of procedures in pharmacopoeias.

Linearity &Calibration Curve

Linearity of an analytical produce is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship test results should be evaluated by appropriate statistical methods for example, by calculation of a regression line by the **method of least squares**. Therefore data from regression line itself may be helpful to provide mathematical estimates of the degree of linearity. For linear ranges the deviations should be equally distributed between positive and negative values.

Range

Range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample including these concentrations for which it has been demonstrated that the analytical procedure has a suitable level of precision accuracy and linearity.

The following minimum specified ranges should be considered.

- For the assay of an active substance or a finished product – 80 – 120% of the test concentration.
- For the content uniformity – 70 – 130% of test.
- For dissolution testing, 20% over specified range.

Eg. If the specifications for the controlled released product cover a region from 20% after the 90% after 24hrs, the validated range – 0-110% of label claim.

- For the determination of an impurity.
- Reporting level of impurity to 12% of specifications.

Limit of Detection

Several approaches depending on whether the procedure is a non-instrumental or instrumental are available.

Based on visual Evaluation

The detection limit is determined by the analysis of samples with known concentration of analyte and by establishing minimum level.

Based on signal to noise ratio

This approach can only be applied to analytical procedure which exhibit base line noise. Determination of signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be detected.

Based on the standard deviation of the response and the slope:

Detection Limit (DL) = 3.3 σ/s

Where σ = the standard deviation of the response

S = the slope of the calibration curve

S is calculated from calibration curve of analyte

Based on standard deviation of Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on calculation curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL and QL. The residual standard deviation of a regression line or the standard deviation of y-intercept of regression line may be used as the standard deviation

Recommended Data

The detection limit and the method used for determining the detection limit should be presented.

Eg. In case of visual method relevant chromatogram is acceptable for justification.

In case of calculation or extrapolation method, it must be subsequently validated by independent analysis of suitable number of samples known to be near or prepared at the detection limit.

Limit of Quantification

Several approaches are there based on whether the procedure is instrumental / non instrumental approaches other than are also acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and be establishing the minimum level at which the analyte can be reliable detected. The quantitation limit is generally determined by the analysis if samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on signal to noise ratio

This approach can only be applied to analytical procedures, which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentrations at which the analyte can be reliably detected and quantified. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit and a ratio of 10:1 for quantitation.

A typical to noise ratio is 10:1.

Based on the standard deviation of the response and the slope

The quantization limit (QL) may be expressed as

$$QL = \frac{10\,\sigma}{S}$$

Where σ = the standard deviation of the response

S = slope is obtained from calibration curve

The estimate may be curried out by various methods

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the calibration curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL and QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Robustness

It is depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

Examples of typical variations are

- Stability of analytical solutions
- Extraction time

In case of liquid chromatography, examples of typical are

- Influence of variation of pH in a mobile phase

- Influence of variation in mobile phase composition
- Different columns
- Temperatures
- Flow rate

In case of gas chromatography

- Different columns
- Temperature
- Flow rate

Ruggedness

Degree of reproducibility of test results obtained by analyzing the same sample under variety of normal test conditions. Such as different

- Analysts
- Instruments
- Days
- Reagents
- Column TLC plates

Comparison of reproducibility of test results to be precision of assay is the direct measure of ruggedness of the method.

2. DRUG PROFILE

TENOFOVIR

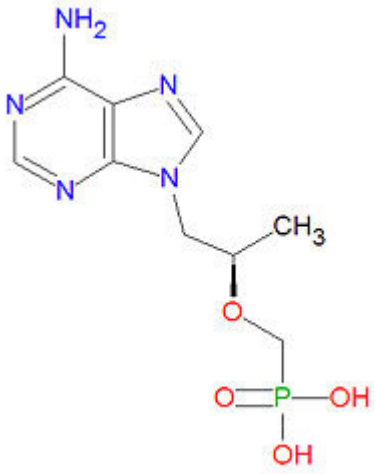
2.1 Chemical properties:

IUPAC name: ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)phosphoric acid

Molecular Weight: 287.213 g/mol

Molecular Formula: $C_9H_{14}N_5O_4P$

STRUCTURE:



2.2 Physical Properties:

Tenofovir is a white to off-white crystalline powder with a solubility of 13.4 mg/mL in distilled water at 25 °C. It has an octanol/phosphate buffer (pH 6.5) partition coefficient (log p) of 1.25 at 25 °C.

2.3 Pharmacokinetic data:

Absorption: In fasted patients is approximately 25%. Administration of food (high fat meal containing 40 to 50% fat) increases the oral bioavailability, with an increase in the AUC of approximately 40%.

Protein binding: < 0.7% to human plasma proteins and < 7.2% to serum proteins.

Half-life: Approximately 17 hours.

Excretion: Excreted renally by glomerular filtration and active tubular secretion

2.4 Metabolism:

Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and after incorporation into DNA by DNA chain termination. Specifically, the drugs are analogues of the naturally occurring

deoxynucleotides needed to synthesize the viral DNA and they compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain. However, unlike the natural deoxynucleotides substrates, NRTIs and NtRTIs (nucleoside/tide reverse transcriptase inhibitors) lack a 3'-hydroxyl group on the deoxyribose moiety. As a result, the next incoming deoxynucleotide cannot form the next 5'-3' phosphodiester bond needed to extend the DNA chain. Thus, when an NRTI or NtRTI is incorporated, viral DNA synthesis is halted, a process known as chain termination. All NRTIs and NtRTIs are classified as competitive substrate inhibitors.

2.5 Uses:

Tenofovir is used with other medications to help control HIV infection, thereby improving quality of life. It helps to decrease the amount of HIV in body so immune system can work better. It also lowers risk of getting HIV disease complications (such as new infections, cancer). Tenofovir belongs to a class of drugs known as nucleotide reverse transcriptase inhibitors (NRTIs).Tenofovir is not a cure for HIV and it does not prevent the spread of HIV to others through sexual contact or blood contamination (such as sharing used needles).

Tenofovir is also used to treat a certain type of liver infection called chronic hepatitis B infection. It helps to decrease the amount of hepatitis B virus in body by interfering with virus growth.

3. REVIEW OF LITERATURE

Kandagal P.B et al[6], 2008 reported A simple reverse-phase high-performance liquid chromatographic method for the determination of tenofovir disoproxil fumarate (TDF) in pharmaceutical formulations and human plasma samples. Piroxicam (PRX) was used as an internal standard. The assay of the drug was performed on a CLC C_{18} (5 µ, 25 cm x 4.6 mm i.d.) with UV detection at 259 nm. The mobile phase consisted of acetonitrile-water mixture in the ratio of 75:25 and a flow rate of 1 ml/min was maintained. The standard curve was linear over the range of 0.2-10 µg/ml (r^2 = 0.9966). Analytic parameters have been evaluated. Within-day and between day precision as expressed by relative standard deviation was found to be less than 2%. The method has been applied successfully for the determination of TDF in spiked human plasma samples and pharmaceutical formulations.

Rezk NL et al[7],2005 described an accurate, sensitive and simple reverse-phase (RP) high-performance liquid chromatography (HPLC) method for the simultaneous quantitative determination of emtricitabine and tenofovir in human blood plasma is described. Using 200 µl of plasma and BOND ELUT-C_{18} Varian columns, the solid phase extraction (SPE) method results in a clean baseline and high extraction efficiencies (100% for emtricitabine and 98.6% for tenofovir). An Atlantis trade mark dC_{18} analytical column was used along with an 18 min linear gradient elution of phosphate buffer (pH 5.7) and methanol to provide sharp peaks for emtricitabine at 280 nm, tenofovir at 259 nm and for the internal standard 2',3'didoxyuridine (DDU) at 262 nm. The method was validated over the range of 10-10,000 ng/ml for both analytes, and was accurate (average accuracies of three different concentrations ranged from 98 to 105% for emtricitabine and 97 to 103% for tenofovir) and precise (within- and between-day precision ranged from 1.7 to 3.7% and 3.7 to 5.2%, respectively). This method could be suitable for use in clinical pharmacokinetic studies and is nimble enough for therapeutic drug monitoring.

El Barkil M et al [8], 2003 developed a sensitive high-performance liquid chromatography method coupled to UV and single mass spectrometry (MS) detection for the determination of tenofovir in human plasma. A solid phase extraction procedure (Bond-Elut C_{18} Varian cartridges) provided high extraction efficiency (91% for tenofovir and 68.8% for the internal standard, 3-methylcytidine). An atlantis-dC_{18} analytical column was used with an isocratic mode elution of a mixture (pH 2.5) of ammonium acetate/methanol (98.5:1.5, v/v). Detection was performed at 260 nm and by using the ion at m/z 288. The signals from both detectors

were validated over the range of 10-1000 ng/ml and were found to be linear, accurate and precise. At the lowest limit of quantification, 10 ng/ml for UV and 5 ng/ ml for MS, the average coefficient of variation was 6.9 and 3.9%, respectively. The potential of this validated method for clinical studies was investigated by analysing more than 170 samples from HIV-infected adult patients. A good correlation was observed between the results obtained with both detectors. However, in several cases discordant results were observed between UV and MS detections. Therefore, tenofovir could suffered from interferences using either UV or single MS detection. They concluded that the double detection allowed to obtain a more specific quantification of tenofovir. The present assay was sound and could be used for therapeutic drug monitoring allowing a higher reliability of the results which are transmitted to the medical team.

Jullien V et al, 2003[9] developed a sensitive high-performance liquid chromatography method with spectrofluorimetric detection for the determination of tenofovir in human plasma. After precipitation of 200 µl of plasma samples by methanol and evaporation of the supernatant, fluorescent derivatized compounds were obtained by a 40-min incubation at 80ºC with chloroacetaldehyde 0.34% at pH 4.5. The assay was performed isocratically using 5 mM Na_2HPO_4 (pH 6), containing tetrabutylammonium (TBA) chloride 5 mM, and acetonitrile (85:15, v/v) as mobile phase and a Cluzeau C_8 plus satisfaction column maintained at 35ºC. Detection was performed at excitation and emission wavelengths set at 236 and 420 nm, respectively. In these conditions, tenofovir could be separated from adefovir, the internal standard and endogenous substances. The method was found to be linear and had been validated over a concentration range of 5-1000 µg/ml. The average coefficient of the limit of quantification (5 µg/ml) was 5.38% and at this concentration, a signal-to-noise ratio of 500 was measured.

Sentenac S et al, 2006[10] reported a new high-performance liquid chromatography assay for the determination of tenofovir, a nucleotide analogue, in plasma. A solid-liquid extraction procedure was coupled with a reversed-phase HPLC system. The mobile phase consisted of Na_2HPO_4 buffer, tetrabutyl ammonium hydrogen sulfate and acetonitrile for different elution through a C_{18} column with UV detection. This method proved to be accurate, precise and linear between 10 and 4000 ng/ml. This method was applied to determine trough levels of tenofovir in 11 HIV-infected patients with virologic failure under multiple antiretroviral therapy. This method was also successfully applied to a pharmacokinetic study in an HIV infected patient with renal failure.

Delahunty T et al, 2005[11] developed and validated an LC/MS/MS method for the determination of tenofovir (TNF) with the beta anti coagulated human plasma matrix. Heparin-treated plasma and serum matrices were also validated. After addition of adefovir as an internal standard, trifluoroacetic acid was used to produce a protein-free extract. Chromatographic separation was achieved with a Polar-RP Synergi, 2.0 mm x 150 mm, reversed-phase analytical column. The mobile phase was 3% acetonitrile/1% acetic acid, aq. Detection of TNF and the internal standard was achieved by ESI MS/MS in the positive ion mode using 288/176 and 274/162 transitions, respectively. This method was linear from 10 to 750 ng/ml with a minimum quantifiable limit of 10 ng/ml when 250 microl aliquots were analyzed. The usefulness of this LC/MS/MS method to routinely monitor plasma concentrations of TNF was demonstrated along with its ability to assist in the performance of pharmacokinetic studies.

Colombo S et al, 2006[12] reported an HPLC method for the determination of the novel non-peptidic HIV protease inhibitor tipranavir (TPV) in human plasma by off-line solid-phase extraction (SPE) followed by HPLC coupled with UV-diode array detection (DAD). After viral inactivation by heat, the plasma was diluted with phosphate buffer (pH 7) and subjected to a SPE on a C_{18} cartridge. Matrix components were eliminated with a solution of 0.1% H_3PO_4 solution neutralised to pH 7 and TPV was eluted with MeOH. The resulting eluate was evaporated and reconstituted in 100 µl MeOH/H2O 50/50. A 40 µl volume was injected onto a Nucleosil C_{18} AB column and TPV was analysed by UV detection at 201 nm using a gradient elution program constituted of MeCN and phosphate buffer adjusted to pH 5.12 and containing 0.02% sodium heptanesulfonate. The calibration curves were linear up to 75 µg/ml, with a lower limit of quantification of 0.125 microg/ml. The mean absolute recovery of TPV is 77.1+/-4.0%. This method was precise with mean inter-day coefficient of variations (CVs) within 2.2-3.4%, and accurate (range of inter-day deviations from 0.7 to 1.2%). This method had been validated and is currently applied to the monitoring of TPV plasma levels in HIV patients.

King T et al, 2007[13] developed a LC -MS-MS method to facilitate the evaluation of drug safety, virologic activity and pharmacokinetics. An anion exchange isolation of tenofovir-diphosphate (TFV-DP) from human peripheral blood mononuclear cells (hPBMCs), coupled with dephosphorylation, desaltation and detection was reported . hPBMCs were harvested from whole blood, lysed and a suspension of intracellular tenofovir moieties was produced. TFV-DP was isolated from TFV-monophosphate (TFV-MP) and tenofovir (TFV)

dephosphorylated with acid phosphatase to form TFV and then desalted and concentrated. The lower limit of quantitation (LLOQ) of the method is 10 fmol per million cells with 5 million hPBMCs used.

Bennetto-Hood Cet al, 2009[14] repoted a sensitive and specific method for the quantitation of tenofovir (TFV) in human plasma by liquid chromatography/electrospray ionization mass spectrometry. Plasma samples were prepared by solid-phase extraction performed on Waters Oasis cation-exchange cartridges (30 mg). Chromatographic separation was performed isocratically on a reversed-phase Waters Atlantis dC_{18} column (2.0x100 mm, 3 mm). The mobile phase consisted of a hydroxylamine/acetic acid buffer (pH 6.75) and methanol (93:7, v/v). The acquisition was performed in selected ion monitoring mode for the protonated molecular ions [M+H]+ of m/z 288.2 for TFV and 212.2 for the internal standard, zalcitibine. This method was fully validated to determine its specificity, recovery, linearity and sensitivity, accuracy and precision. The analytical range was set at 1-750 ng/ml using a 200 µl plasma sample with a mean coefficient of determination (r2) of 0.9969. The mean accuracies for the calibration standards ranged from -5.0 to 4.3%, while the precisions were within 1.2 and 6.4%. Intra-assay and inter-assay mean accuracies for three quality control concentrations (2, 60, and 600 ng/ml) ranged from -6.1 to 10.7%, while the precisions were within 1.3 and 9.1%. TFV was shown to be stable under normal storage and assay conditions; no degradation was seen when stored at -20 degrees C or -80 degrees C for up to 6 months, and after 16 h at room temperature in the injection matrix. The present method provided an accurate, precise, and sensitive tool for TFV quantitation and was successfully applied to an external proficiency-testing program and pharmacokinetic analysis.

Yadav M et al,2009[15] developed a simple, specific, and high throughput liquid chromatography tandem mass spectrometry method for the determination of tenofovir in human plasma using adefovir as internal standard. Plasma samples were prepared by solid-phase extraction of the analyte and internal standard using Waters Oasis MCX cartridges (1 cc, 30 mg). The chromatographic separation was achieved on a reversed-phase Chromolith, C_{18} analytical column (100 mmx4.6 mm, 5 µm) under isocratic conditions. The mobile phase consisted of 0.5% formic acid in water and acetonitrile (90:10, v/v) to give a run time of 1.8 min. The protonated precursor-->product ion transitions for tenofovir and IS were monitored on a triple quadrupole mass spectrometer operating in the multiple reaction monitoring and positive ion mode. The fragmentation pathways for tenofovir were studied by varying the collision energy (5-55 V) using nitrogen as CAD gas. A linear dynamic range of 3.1-1002.0

ng/ml was established using 0.2 ml plasma sample. The method was fully validated for its sensitivity, selectivity, accuracy, precision, matrix effect, recovery, stability and dilution integrity. It was applied to a bioequivalence study in 43 human subjects after oral administration of 300 mg tablet formulation under fasting conditions.

Ahmed Z et al,2009[16] developed and validated a simple, selective, linear, accurate and precise RP-HPLC method for routine analysis of tenofovir disoproxil in bulk and in pharmaceutical formulation. Isocratic elution at a flow rate of 1.0 mL/min was employed on a Thermo Hypersil HIGH PURITY C_{18} reversed-phase column (150 mm x 2.1 mm i.d., 5 mu m) at ambient temperature. The mobile phase consisted of acetonitrile and 0.05 M phosphate buffer pH 6.0 (50: 50, v/v). The UV detection wavelength was 260 nm and 20 µl of sample was injected. The retention time for tenofovir disoproxil was 4.45 min. The sensitivity, accuracy, precision, robustness, stability, specificity, selectivity and system suitability parameters were validated for this method.

Guo J et al,2002[17] reported a simple, specific, sensitive LC/MS/MS method for the quantitation of tenofovir (TFV) in monkey plasma. After the addition of adefovir as an internal standard (IS), methanol was used to produce a protein-free extract. Isocratic chromatographic separation was performed on a reverse-phase Discovery C(18) column (4.6×250 mm, 5 µm). The mobile phase consisted of methanol-water-formic acid (20 : 80 : 0.5, v/v/v). Detection of TFV and the IS was achieved with electrospray ionization (ESI)-MS/MS in the positive ion mode using 288/176 and 274/162 transitions respectively. The analytical range was set at 0.005-1.250 µg/ml using a 200 µl plasma sample. The intra- and inter-day precision values were less than 11.4% and accuracy ranged from 0.4 to 2.9% in all quality control samples. This method was fully validated for its sensitivity, selectivity, accuracy, precision, matrix effect, recovery and stability. Due to the high polarity of TFV, the major challenge was to circumvent ion suppression when quantitating the plasma concentration of TFV using the LC/MS/MS method. Moreover, it was found that the reconstitution solvents of the dried residue had a significant impact on LC peak shapes. This validated method was successfully applied to a bioequivalence study in 6 monkeys after the oral administration of two ester prodrugs of TFV (equivalent to TFV 20 mg/kg). This method permitted laboratory scientists with access to the appropriate instrumentation to perform rapid TFV determination.

4. AIM AND PLAN OF THE STUDY

4.1 AIM AND SCOPE:

Tenofovir is relatively a newer antiretroviral drug and used in the treatment of HIV.

For quantification of Tenofovir in tablet formulation, very few HPLC methods were available.

Hence the aim of present work is to develop simple and validated RPHPLC method by isocratic mode for the quantification of Tenofovir in bulk and it's formulation.

4.2 PLAN OF WORK:

The plan of present work is as follows:

Method Development

- Selection of wavelength
- Selection of initial separation conditions
- Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
- Nature of the stationary phase
- Selection of separation method and agent

Validation of the developed method

The developed method has to be validated by using the various validation parameters such as,

- Accuracy
- Precision
- Linearity and of detection (LOD) / Limit of quantitation (LOQ)
- Selectivity / Specify
- Robustness / ruggedness
- System suitability.

5. METHODOLOGY

5.1 MATERIALS AND INSTRUMENTS USED

a) Drug samples:

Tenofovir pure drug sample was obtained from Hetero labs, Hyderabad and tablet dosage form belongs to Ranbaxy pharma(Tentide - 300 mg).

b) Chemicals and Solvents used:

S. No	Name	Grade
1.	Methanol	Merck (HPLC Grade)
2.	KH_2PO_4	HPLC Grade
3.	Acetonitrile	Merck (HPLC Grade)
4.	Tetrahydrofuron	AR grade

c) Instruments used:

S. No	Name	Model
1.	HPLC	Peak 7000
2.	Digisun pH meter	2001
3.	Eletronic balance	ELB 300
4.	UV Spectrophotometer	2201 UV-VIS

5.2 OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR THE ESTIMATION OF TENOFOVIR

The present work is for the development of the RP-HPLC method for the Tenofovir and its validation.

5.2.1 Selection of wavelength

An UV spectrum of 100µg/ml Tenofovir in methanol was recorded by scanning in the range of 240 nm to 270 nm. From the absorbance data 260 nm was selected **.** At this wavelength Tenofovir showed good absorbance **table.1**.

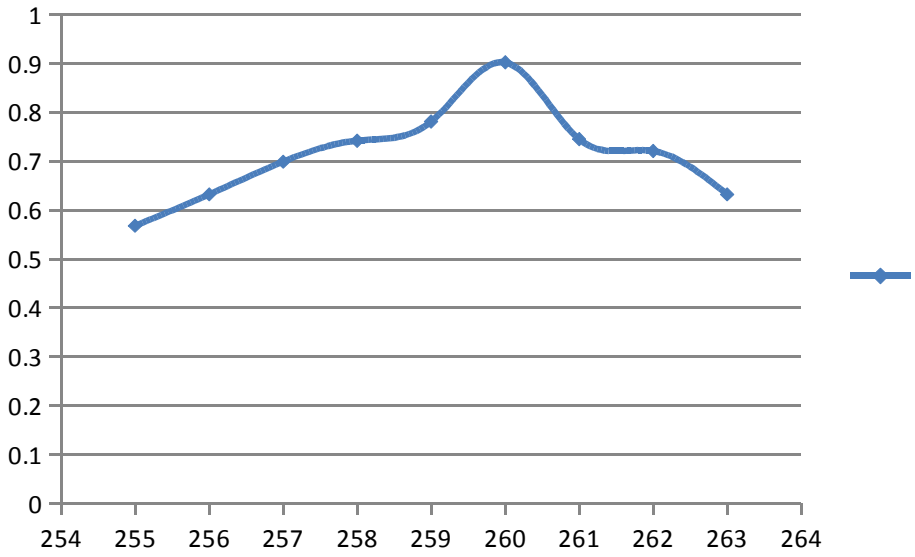


Fig 2: UV Absorbance graph.

Table 1: Data of Absorbance

S. No.	Wavelength	Absorbance
1.	255	0.568
2.	256	0.632
3.	257	0.699
4.	258	0.742
5.	259	0.781
6.	**260**	**0.902**
7.	261	0.745
8.	262	0.721
9	263	0.632

5.2.2 Selection of chromatographic method

Proper selection of the method depends upon the nature of the sample (ionic,ionisable,neutral molecule), its molecular weight and solubility. The drug selected in the present study is polar in nature and hence reverse phase or ion-pair or ion–exchange chromatography method may be used. The reverse phase HPLC was selected for the initial separations because of its simplicity and suitability.

Initial separation conditions

Standard solution : 100 µg/ml of Tenofovir in HPLC grade methanol.

Equipment

System : PEAK7000 isocratic HPLC

Pump : PEAK 7000 delivery system

Detector : Diode array detector

Injector : Rheodyne 7725*i* with 20 µl loop

Trail 1:

Chromatographic conditions:

Mobile phase :Methanol: Acetonitrile (50+50)

Column :C_{18} 250 x 4.6mm

Flow rate :1.0ml/min

Runtime :10min

P^H : 4.8 by H_3PO_4

Wave length :262nm

Sample size : 20 µl

Needle wash : water HPLC grade

Column temperature : ambient

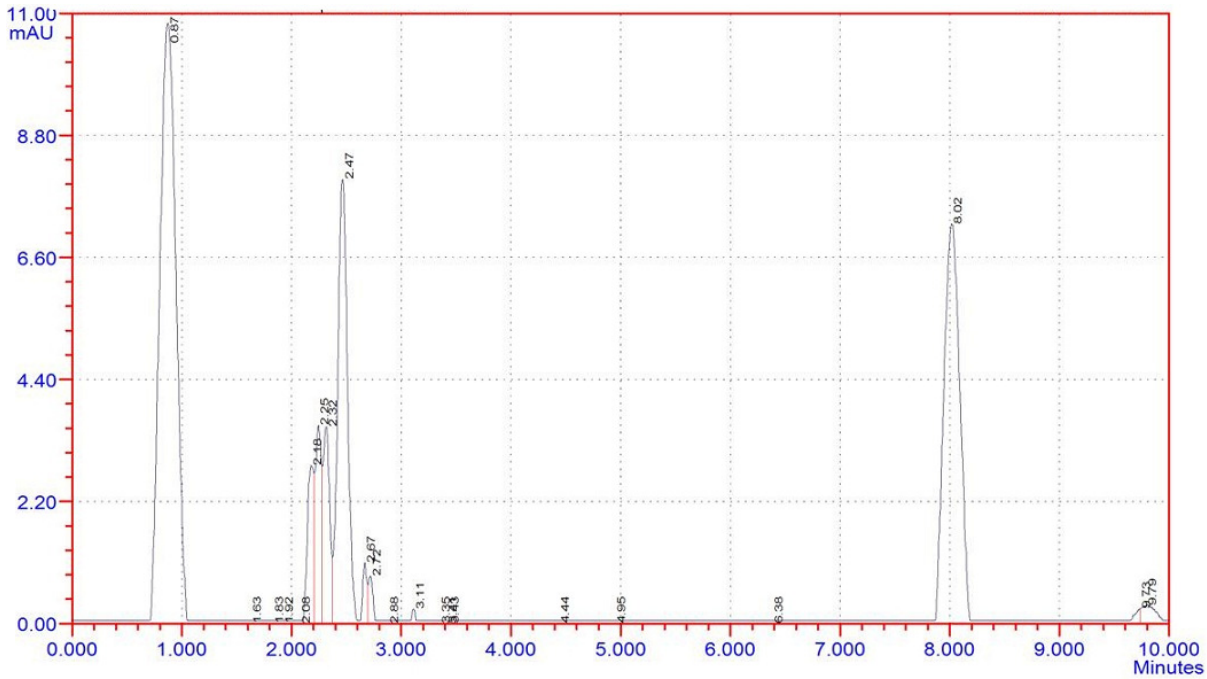


Fig 3: trail 1

Observation: There was no proper separation and merging of peaks was appeared.

Trial 2:

Chromatographic conditions:

Mobile phase	:Methanol: Water (90+10)
Column	:C_{18} 250 x 4.6mm
Flow rate	:1.0ml/min
Runtime	:10min
P^H	:5.2 by H_3PO_4
Wave length	:262nm
Sample size	: 20 µl
Needle wash	: water HPLC grade
Column temperature	: Ambient

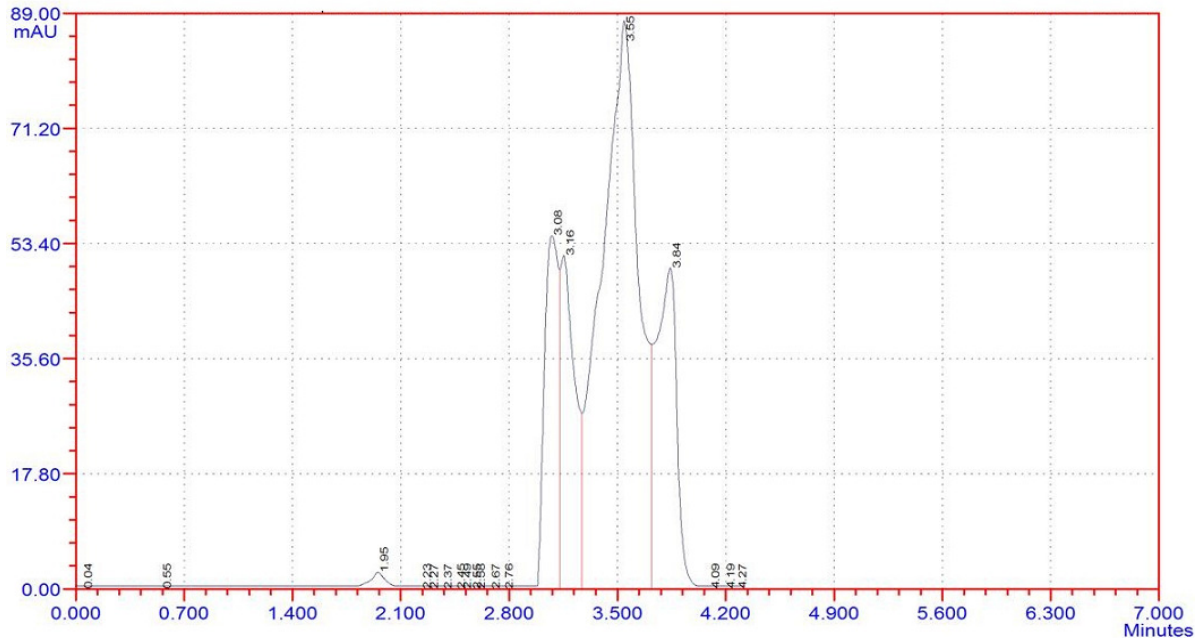


Fig 4: trail 2

Observation: merging and no symmetry of peaks.

Trial 3:

Chromatographic conditions:

Mobile phase	: Methanol: KH2Po4 (0.01M) (50+50)
Column	: C_{18} 250 x 4.6mm
Flow rate	: 1.0ml/min
Runtime	: 7min
P^H	: 5.6 by H_3PO_4
Wave length	: 262nm
Sample size	: 20 µl

Needle wash : water HPLC grade

Column temperature : ambient

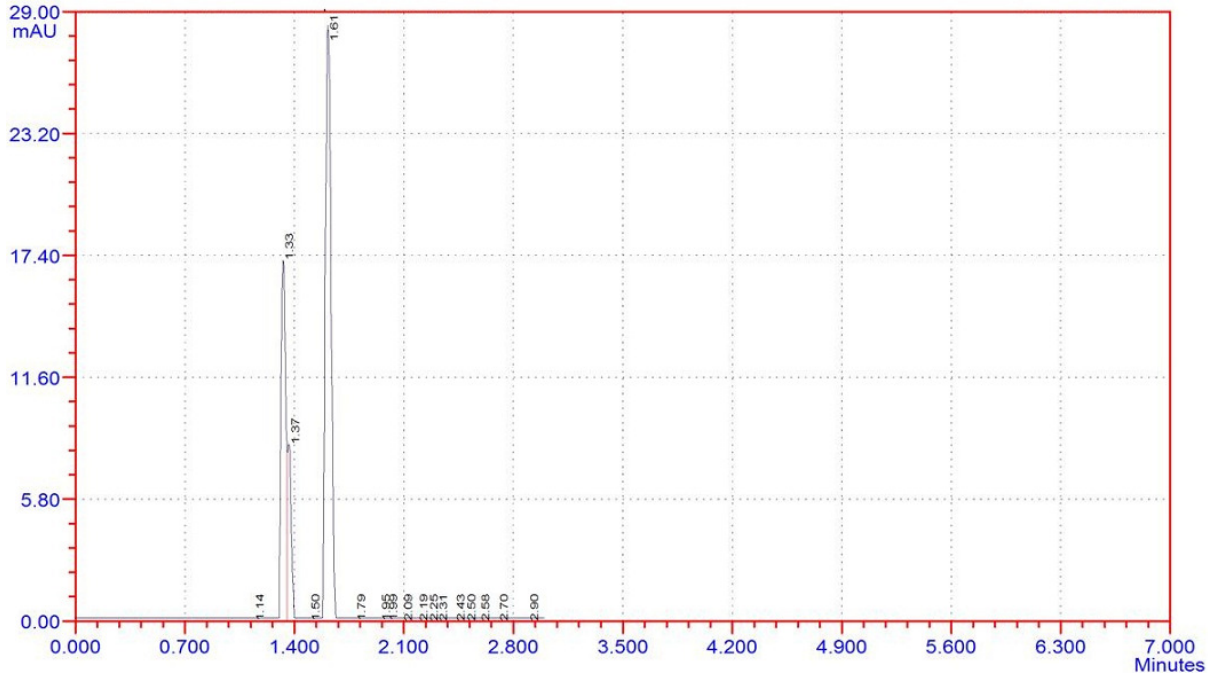


Fig 5: trail 3

Observation: merging of peaks and no proper peak

Trial 4:

Chromatographic conditions:

Mobile phase :Methanol: KH2Po4 (0.01M): Acetonitrile (50+25+25)

Column :C_{18} 250 x 4.6mm

Flow rate :1.0ml/min

Runtime :6 min

P^H :4.9 by H_3PO_4

Wave length :260nm

Sample size : 20 µl

Needle wash : water HPLC grade

Column temperature : ambient

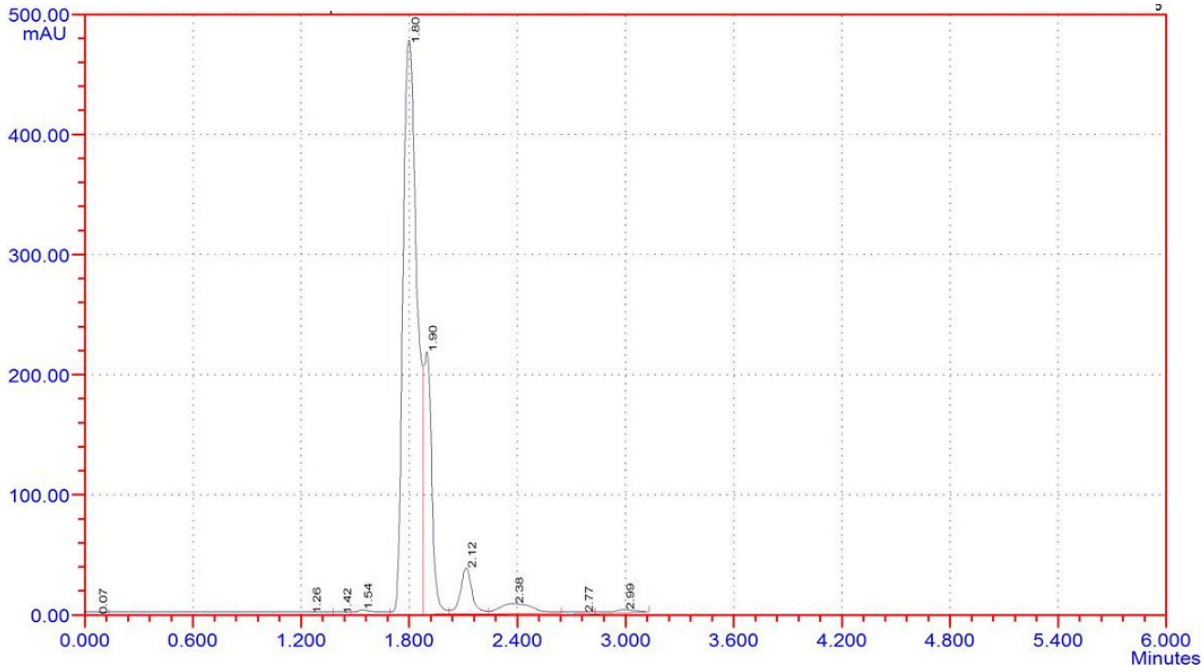


Fig 6: trail 4

Observation: Symmetrical Peak was not observed.

OPTIMIZED METHOD

Fixed chromatographic condition:

Detection:	260 nm
Mobile phase:	Methanol: kH_2PO_4 (0.01M):THF: Acetonitrile (55+10+5+30)
Stationary phase:	C_{18} 250 x 4.6mm
Flow rate:	1.2ml/min
Runtime:	10min
Retention time:	3.7 min
P^H :	5.5 by H_3PO_4
Sample size:	20 µl
Needle wash :	water HPLC grade
Column temperature:	room temperature (20ºC)

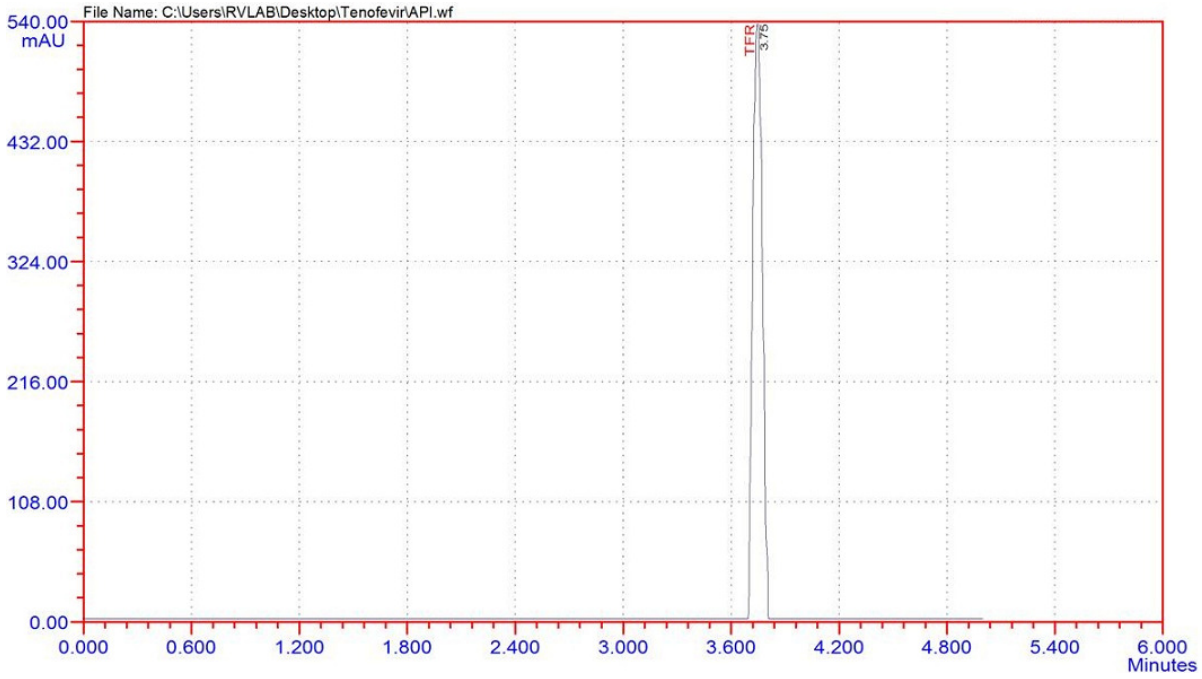


Fig 7: Fixed Chromatographic Condition

Observation:

Peak shape was good, theoretical plates were good and asymmetry was within the limit.

5.3 QUANTIFICATION STUDY

Preparation of Standard Solution:

1 gram of TENOFEVIR A.P.I. (pure drug form) was taken and dissolved in methanol and finally made the volume to 100ml in volumetric flask with methanol. Then it was suitably diluted with methanol to produce final drug concentration of 100µg/ml.

Preparation of Sample Solution:

The formulation tablets of Tenofevir (Tentide - 300 mg) were crushed to give finely powdered material. Then tablet powder equivalent to 100 mg of tenofovir was transferred into 100 ml flask and volume was made up with mobile phase and filtered through Ultipor N_{66} Nylon 6, 6 membrane sample filter paper. Filtrate was suitably diluted with mobile phase to produe drug concentration of 100µg/ml.

Recording of chromatogram:

With the optimized chromatographic conditions mentioned above, a steady baseline for about 20 min was obtained. After the stabilization of the baseline for about 20 min, the

standard and sample solutions were injected separately into chromatographic system and chromatogram was recorded until the reproducibility of the peak area was found satisfactory. Retention time of Tenofovir was found to be 3.7mins**.**

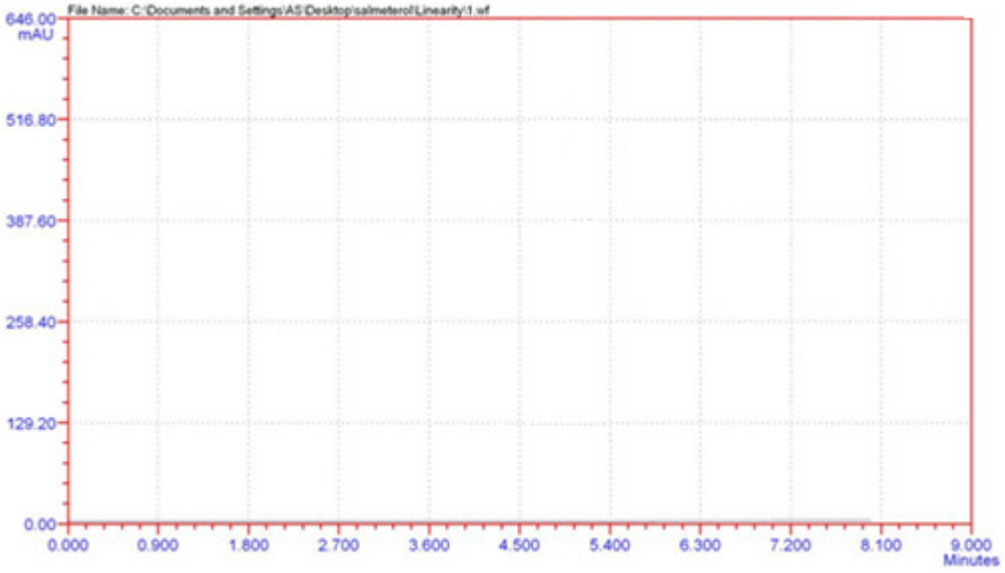


Fig 8: Chromatogram of Blank

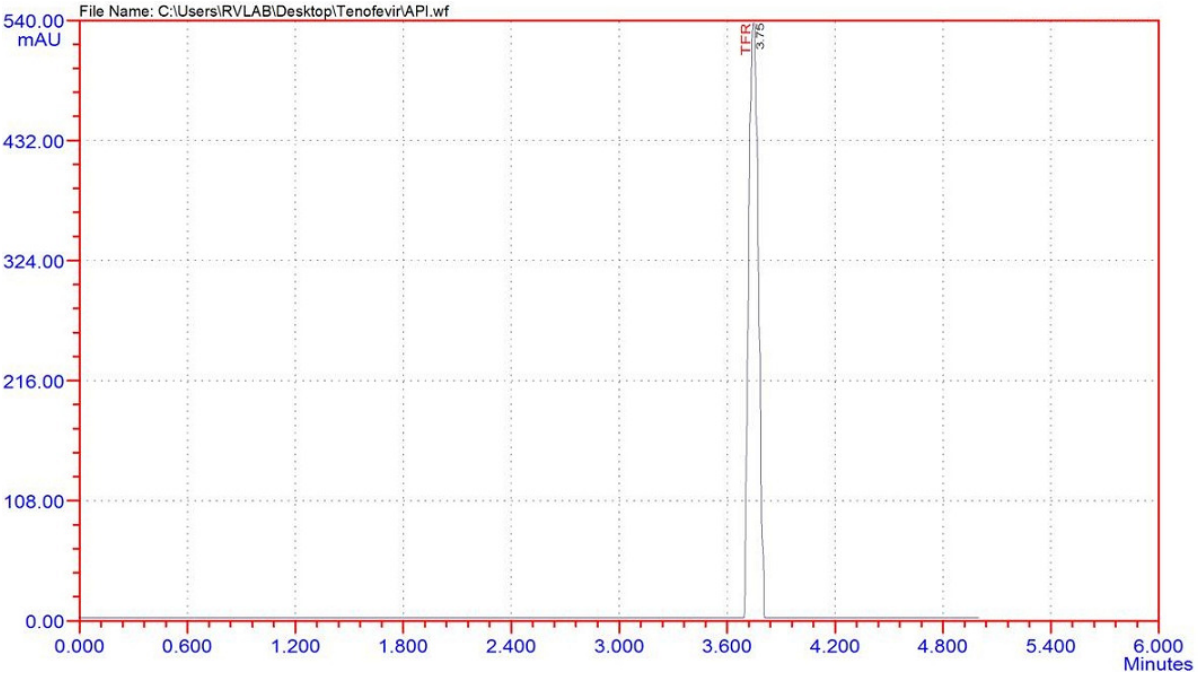


Fig 9: Chromatogram of standard

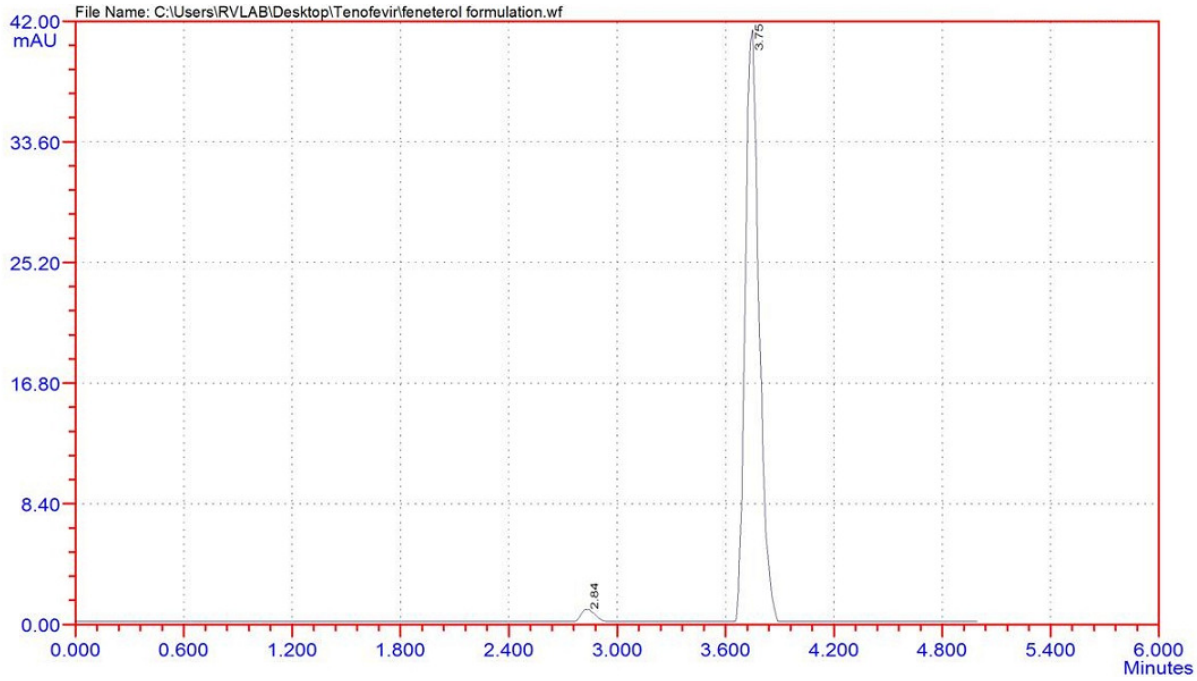


Fig 10: Chromatogram of sample

5.4 VALIDATION OF THE METHOD

After the development of HPLC method for the estimation of Tenofovir in a dosage form, validation of the method was performed. This section described the procedure followed for validation of the developed method.

1) Accuracy:

Accuracy of the method was determined by standard addition method. A known amount of standard drug was added to the fixed amount of pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 50%, 100% and 150% level of 100ppm. The solutions were analyzed in triplicate at each level as per the proposed method. The percent recovery and % RSD was calculated and results are Satisfactory. Recoveries ranging from 99.0 to 100.1 were obtained by the proposed method. This indicates that the proposed method was accurate.

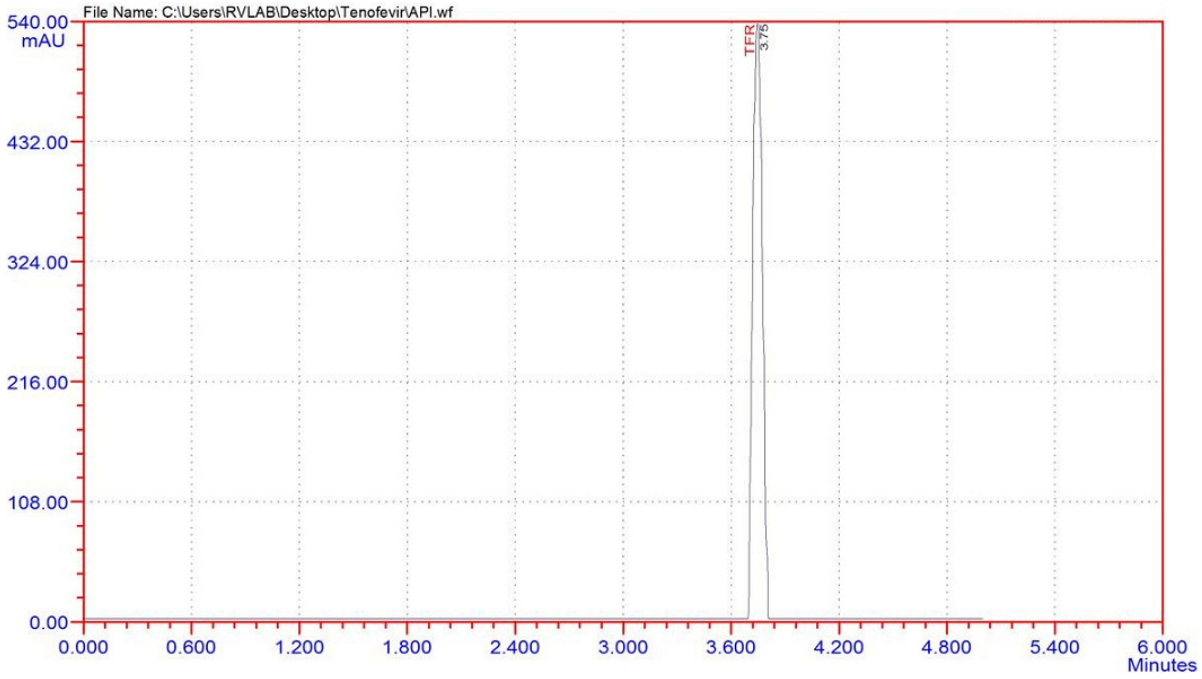


Fig 11: Chromatogram of accuracy(a).

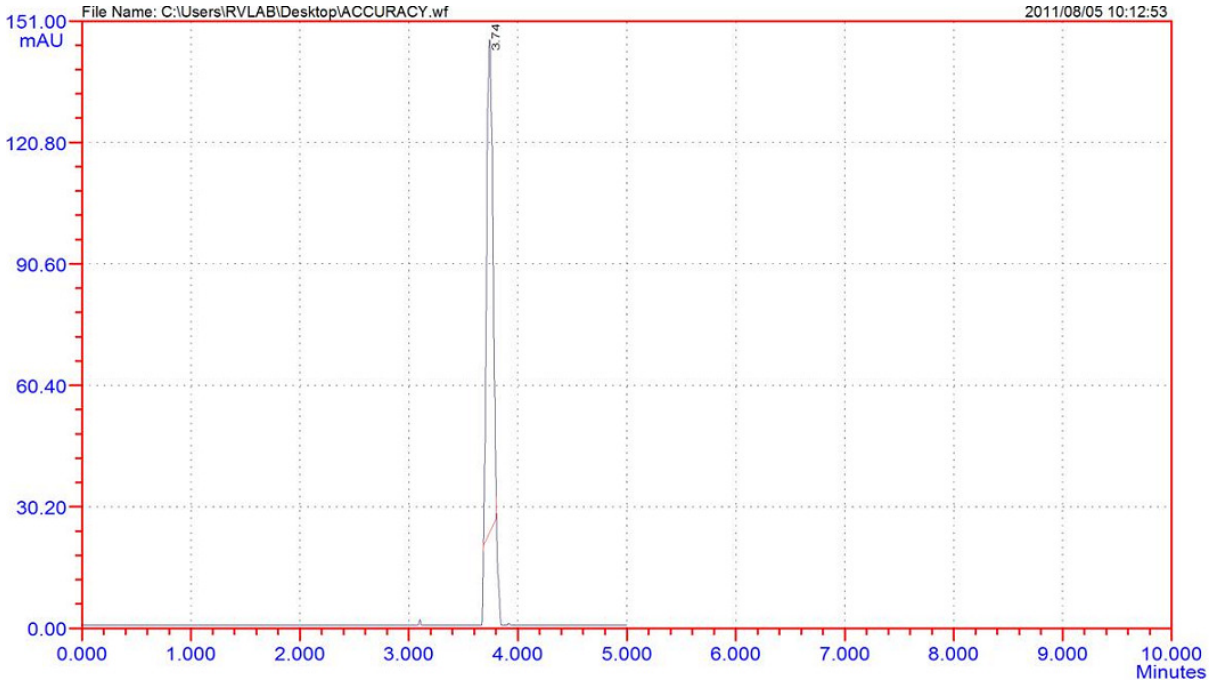


Fig 12: Chromatogram of accuracy(b).

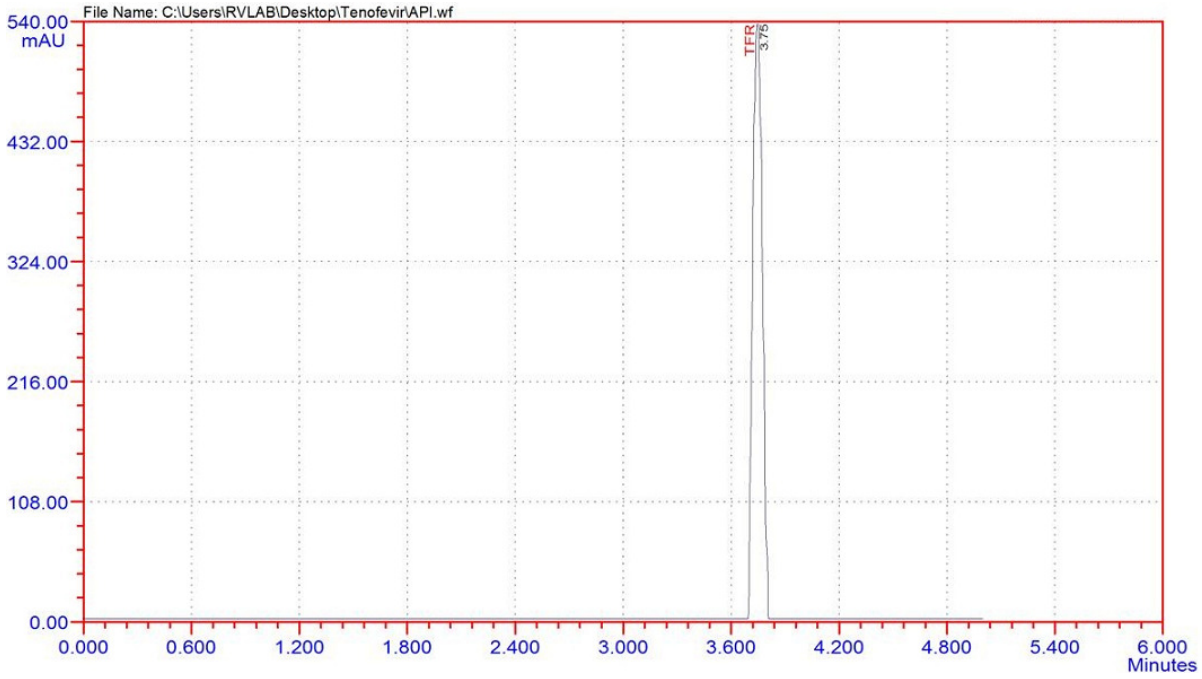


Fig 13: Chromatogram of accuracy(c).

Table 2: Data of accuracy

Level	Amount of Tenofevir spiked (µg)	Amount of recovered(µg)	% Recovery	%RSD
50 %	150	149	99.33	0.166
	150	149.5	99.66	
	150	149.2	99.46	
100%	200	198.9	99.45	0.125
	200	199.2	99.6	
	200	199.4	99.7	
150%	250	249.3	99.72	0.069
	250	249.3	99.72	
	250	249.0	99.6	
			Mean % of recovery 99.58	Mean RSD =0.12

Acceptance Criteria:

For an assay method, mean recovery should be 100% ± 2% at each concentration over the range of 50-150% of the target concentration.

2) Precision:

Repeatability and reproducibility studies were conducted to determine precision of the method. Repeatability studies were done by consequently injecting the standard solution of same concentration i.e.,100 µg/ml of Tenofovir. The solutions were prepared and injected as per assay procedure.

Fig 14: Chromatogram of precision

Table 3: Data of repeatability

Inj. No	Peak Area
1.	24389
2.	24358
3.	24366
4.	24405
5.	24327
Avg	24369
SD	29.958
% RSD	0.1229

Intraday Studies

Intraday studies were conducted to determine the solution stability. Intraday studies were done by injecting the standard solution of Tenofovir at different intervals of time and the data was shown below:

Table 3(a):Intra day

Inj. No	Conc. of Efavirenz (µg/ml)	Time Intervel(hrs)	Peak Area	% RSD
1	100	0	24389	
2	100	6	24266	0.3770
3	100	12	24210	

Inter day Studies

Inter day studies were conducted to determine the solution stability in different days. Inter day studies were done by injecting the standard solution of Tenofovir in different days (Day1,Day2) and the data was shown below:

Table 3(b): Inter day Studies

No of Injection	Conc. of Tenofovir (µg/ml)	Peak Area	% RSD
Day 1	100	24389 24337 24256	0.286
Day 2	100	24227 24111 24132	0.255

Acceptance criteria

The % Relative Standard Deviation of peak areas of Tenofovir should not be more than 2.0.

3) Linearity and Range:

From the standard stock solutions, a suitable standard solution was prepared to contain targeted level of the assay concentration of the standard drug. The solutions were examined by the assay procedure. The standard solution of Tenofovir containing 100-500µg/ml was injected and chromatogram was recorded and were appended in **figure no 15-20**. The calibration curve was plotted using peak area Vs concentration of the standard solution. From the calibration curve, the slope and intercept were calculated **fig 20.**

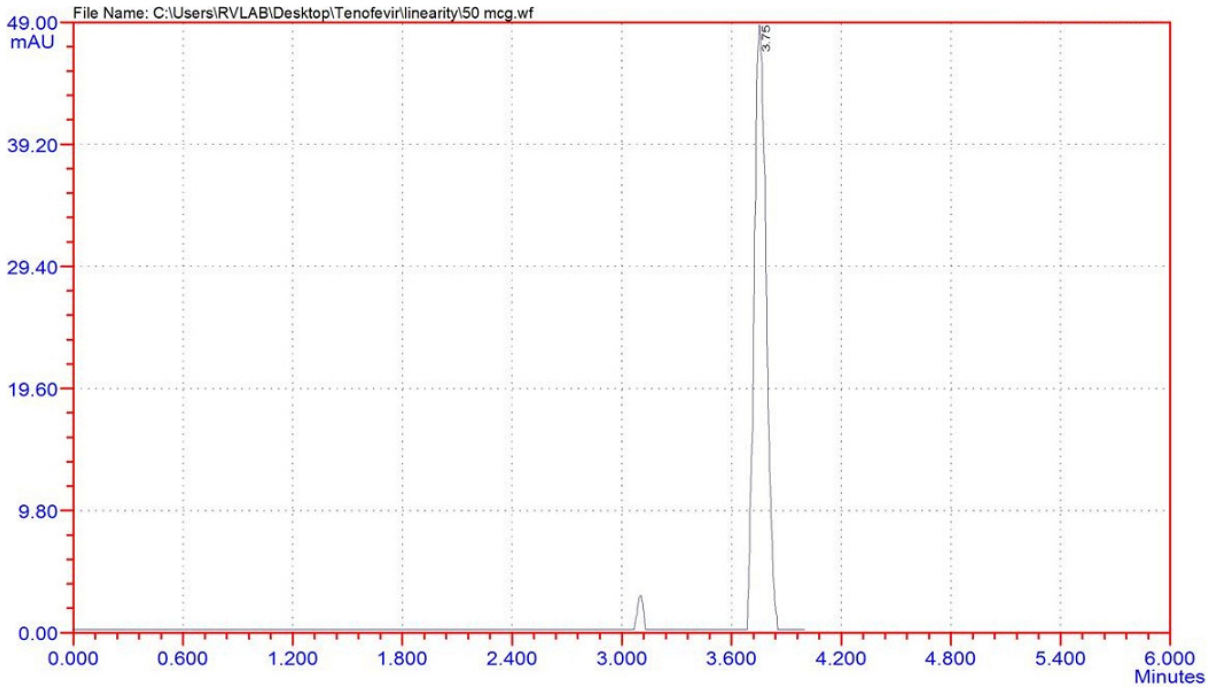


Fig 15: Chromatogram of Tenofovir 50µg/ml

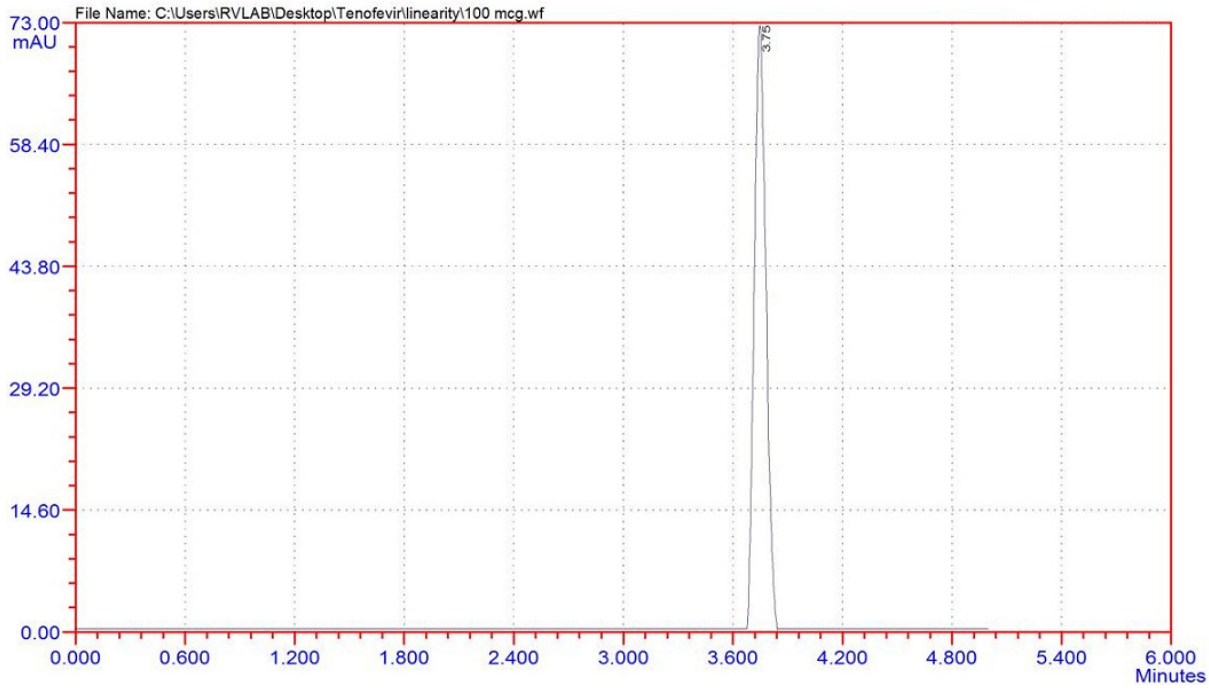


Fig 16: Chromatogram of Tenofovir 100µg/ml

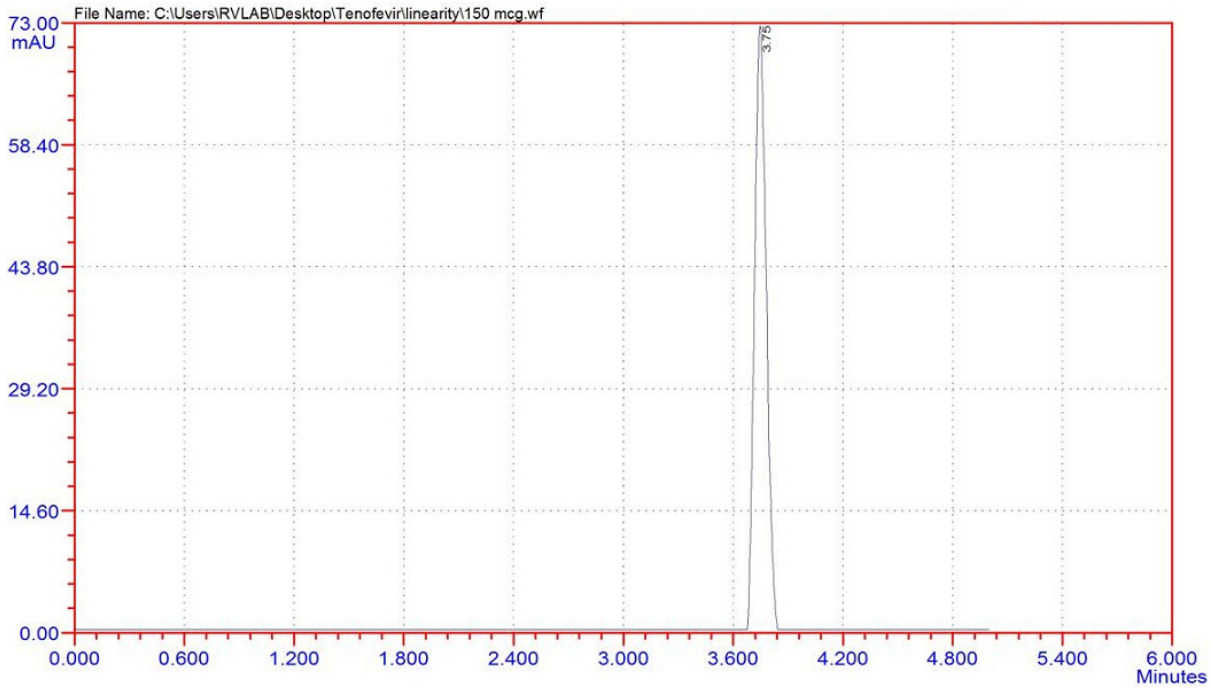


Fig 17: Chromatogram of Tenofovir 150µg/ml

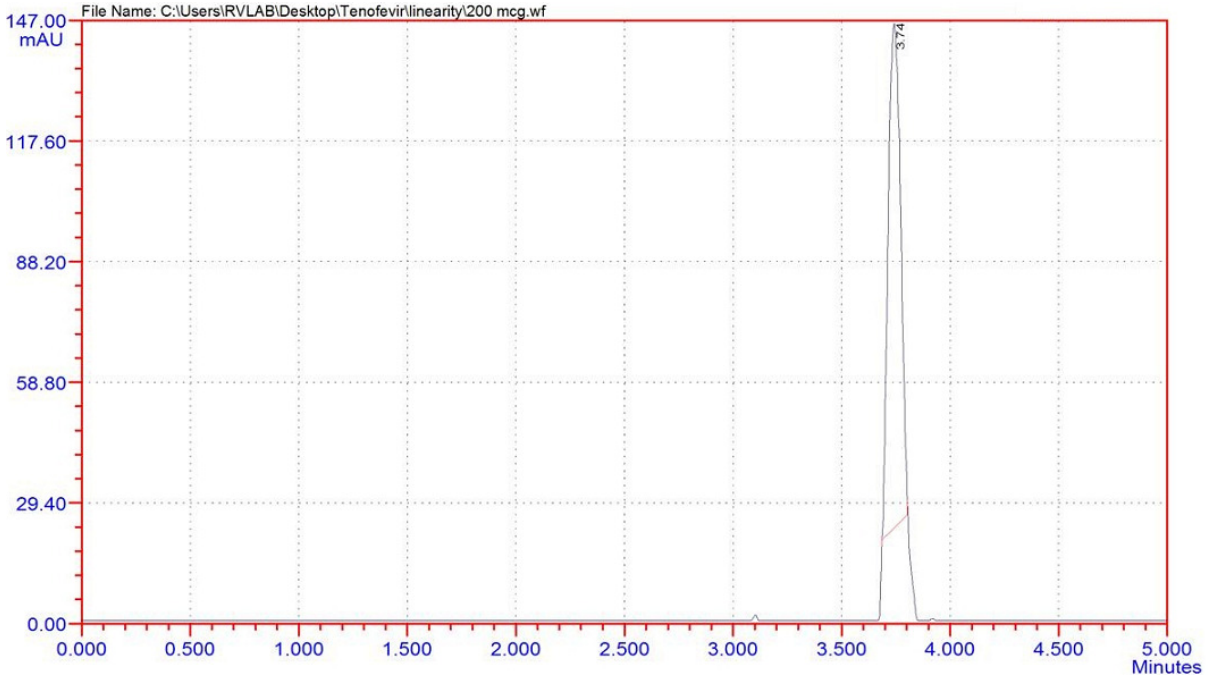


Fig 18: Chromatogram of Tenofovir 200µg/ml

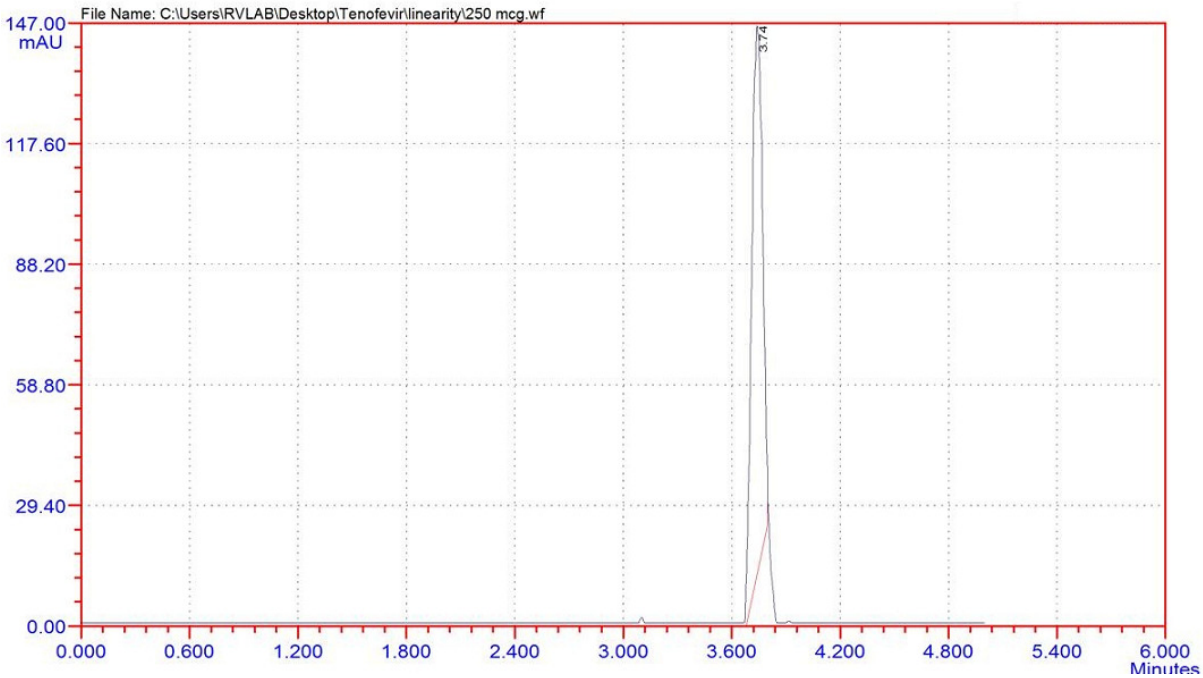


Fig 19: Chromatogram of Tenofovir 250µg/ml

Table 4: Data of linearity

CONC	AREA	T.PLATES
50 µg	12246	10196
100 µg	24389	13211
150 µg	35038	10012
200 µg	46577	17945
250 µg	57734	15530

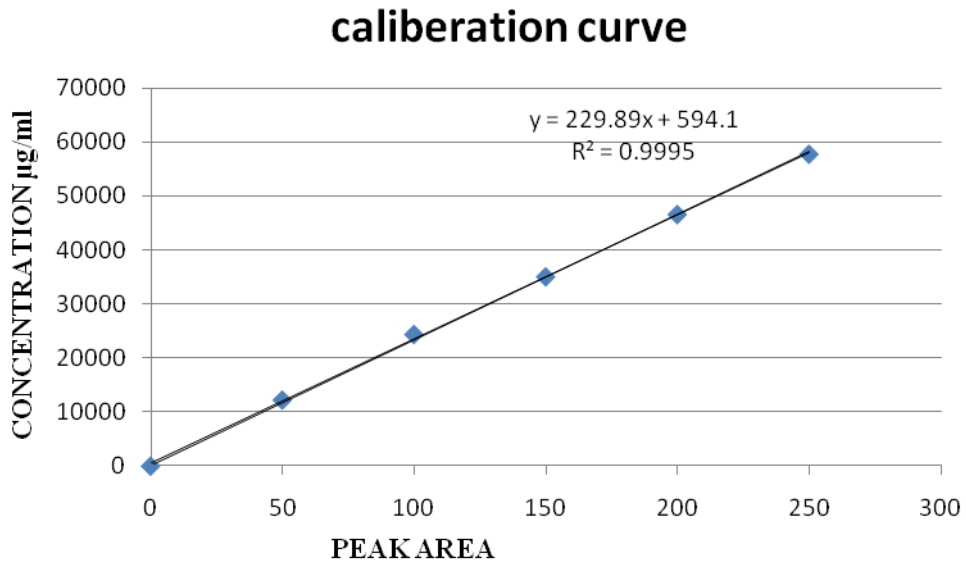


Fig 20: caliberation curve

Acceptance Criteria

Coefficient of correlation should be not less than 0.999

4) Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD and LOQ of the developed method were determined by analyzing progressively low concentration of the standard solutions using the developed methods. The LOD is the smallest concentration of the analyte that gives a measurable response . LOD of Tenofovir was found to be 15 µg/ml. The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Tenofovir was found to be 20 µg/ml.

5) Specificity

The specificity of the RP-HPLC method was determined by complete separation of Tenofovir as shown in above Figures with parameters like retention time (t_R), resolution (Rs) and tailing factor (T), peak purity curve and peak purity index. Tailing factor for peaks of Tenofovir was less than 2% and resolution was satisfactory. The average retention time ± standard deviation for Tenofovir was found to be 4.1±0.0148 for six replicates. The peaks obtained for Tenofovir was sharp and have clear baseline separation. The peak purity studies were performed to prove that the method is specific in nature.

6) System Suitability Studies:

The system suitability studies were carried out as specified in USP. These parameters include column efficiency, resolution and capacity factor.

Table 5: System Suitability Parameters

Validation Parameters	Acceptance Criteria
Linearity range (µg/ml)	50 - 250
Correlation co-efficient (r^2)	0.999
LOD (µg/ml)	15
LOQ (µg/ml)	20
Intraday (%RSD)*	0.377
Interday (%RSD)*	0.281
Repeatability (%RSD)*	0.1229
Accuracy (%)	99.32 - 99.72
Peak purity index	1.0000
Resolution factor (Rs)	-
No. of theoretical plates (N)	24389
Tailing factor	1.14

7) Robustness:

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

1) ± 2% in ratio of Methanol in mobile phase,
2) ± 0.1 ml of flow rate,
3) ± 0.2 units in the pH.

The separation factor, retention times and peak symmetry were then calculated.The deviation among the results obtained is well within the limits. Hence the method is robusted.

Table 6: Robustness Studies

Chromatographic Condition	Retention time	% Accuracy
Actual concentration	3.747	99.72
Wavelength 261	3.932	99.66
Methanol ratio 40%	2.761	99.45
Flow rate 1.2 ml/min	3.215	99.33

6. RESULTS AND DISCUSSION

The RP-HPLC method was developed for estimation of Efavirenz in tablet dosage form and validation was performed in accordance with ICH guidelines for the following parameters such as system suitability,linearity and range, precision, accuracy, ruggedness, specificity and robustness. The summary of results obtained in analytical method development and validation were shown in table 9.

Table 9: Data and Results

Parameters	Acceptance	Results
System suitability	1. % RSD NMT 2% 2. Tailing factor NMT 2 3. Theoretical plates NLT 2000	0.12 % 1.12 passes
Specificity	No interference with blank	No Interference
Precision (Repeatability)	% RSD NMT 2%	0.1229
Accuracy	98-103%	99.58
Linearity	Correlation co-efficient NLT 0.999	0.9994
Robustness	System suitability parameters should be with in limit NMT 2%	Passes

Validation summary report:

The observations and results obtained for each of the parameters like system suitability, linearity and range, precision (repeatability), intermediate precision, specificity, ruggedness and robustness lies well within the acceptance criteria. So the given method was simple, specific, linear, precise, accurate, robust and rugged and extensively used for the estimation tenofovir in tablet dosage form.

Discussion:

The Reverse Phase High Performance Liquid Chromatography method was developed a stability indicating assay method. Pure drug chromatogram was run in different mobile phases containing methanol, water, acetonitrile, tetrahydrofuron and also by using orthophosphoric acid. Different columns like C_8 & C_{18} were used. The retention time and tailing factor were calculated. Finally the mobile phase with Methanol: kH2PO4 (0.01M):THF: Acetonitrile in the ratio of 55:10:5:30 whose P^H is adjusted to 5.5 using orthophosphoric acid. Kromosil C_{18} 250 x 4.6mm analytical column was selected which gives a sharp peak and a symmetrical peak with 1.14 tailing. Calibration curve was found to be linear at range 50µg/ml to 250 µg/ml. Five different concentrations of Tenofovir in range above were prepared and 20µl of each concentration injected in HPLC. The slope(m) and intercept (c) obtained were found to be 222.89 and 594.1.The correlation of coefficient (r^2)obtained was found to be 0.9995.It was observed that the concentration range showed a good relationship. The limit of detection for Tenofovir was found to be 0.43µg/ml and the limit of quantification was found to be 1.33µg/ml. It proved the sensitivity of the method. The percentage assay or average amount of Tenofovir in formulation was found to be 79.8%.The low values of standard deviation and coefficient of variation at each level of the recovery experiment indicate high precision of the method.

7. CONCLUSION

From the experimental data results and parameters it was concluded that the developed RP-HPLC method has the following advantages:

- The standard and sample preparation requires less time
- No tedious extraction procedure was involved in the analytical process.
- Suitable for the analysis of raw materials.
- Run time required for recording chromatograms were less than 10 minutes
- Hence, the chromatographic method developed for Tenofovir was found to be simple, precise, accurate and cost effective and it could be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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